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Antiserum Preparation For Immunodiffusion In Southern Pine Beetle Predation Studies

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SUMMARY

An anti-adult southern pine beetle serum was produced by subcutaneous injection of rabbits with southern pine beetle (SPB) adult antigen. Initial tests demonstrated the ability of the anti-adult SPB serum to detect adult SPB antigen in the body of the adult predator, *Thanasimus dubius* (F.). Cross reactivity was found between the anti-adult serum and extracts of immature stages of *Dendroctonus frontalis* Zimmerman, adult *D. terebrans* (Olivier), *Ips grandicollis* (Eichhoff), and *I. calligraphus* (Germar).

Adult southern pine beetle protein can be detected in a whole predator extract at least 28 hours after feeding.

Additional keywords: *Dendroctonus frontalis, Thanasimus dubius,* Agar gel double diffusion, precipitin test, predation.

STUDY OF PREDATORS

Through most of its life cycle the southern pine beetle (SPB), *Dendroctonus frontalis* Zimmermann, is a cryptic insect. Except for the adult stage, SPB life cycle stages are hidden beneath the bark of the host tree. A sizeable SPB insect-associate complex exists (Moser and others 1971), but direct measurements of successful attack by SPB natural enemies have not been possible. Accepted methods of estimating insect predation are indirect (Berryman 1967). Identification of biological roles of insect associates of the SPB and related bark beetles has been limited to small-scale, time-consuming rearings and to laboratory or field observations. Work has concentrated mainly on adult stages of those predators easy to obtain, rear, or maintain in the laboratory (Thatcher and Pickard 1966, Berryman 1967, Amman 1970, 1972, Moore 1972, Nagal and Fitzgerald 1975). Few adult insect associates have been studied in a way that provides information regarding their direct impact on the SPB population (Thatcher and Pickard 1966).

Precipitin reactions provide a means for determining the biological roles of predatory insects and mites by indicating their food preferences (West 1950, Dempster 1960, Dasgupta and Cunliffe 1970). These reactions also provide a means for measuring the minimum number of prey consumed (Dempster 1960, Kiritani and Dempster 1973).

This study concerns preparation of an anti-adult southern pine beetle serum, determination of sensitivity and selectivity of the antiserum, and development of a laboratory feeding test for SPB predators.

MATERIALS AND METHODS

Antigen preparation

Newly emerged SPB adults were collected in an emergence box and refrigerated collector similar to that of Browne (1972). Beetles were starved for 24 h, screened, and hand sorted to remove debris. They were washed with distilled water, dried on paper towelling, and crushed in equal volumes of 0.85 percent NaCl in a mortar with a pestle. To inhibit melanization, a few drops of 0.001 M KCN were added to the resulting mixture (Dempster 1960), which was then

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stored at 4°C in 30-ml portions for 24 h. Gross beetle fragments were removed by filtration through glass wool and cheesecloth. When filtering was completed, the glass wool was compressed to extract the liquid, which was then centrifuged at 23,500 *g* for 30 min. at 4°C, and filtered sequentially through Gelman Metrica[TM1/] 5.0, 1.2, 0.8, 0.45, and 0.20 um pore filters. Before the liquid was sterilized with the 0.20 um filter, a sample of supernatant was taken for protein determination (Lowry and others 1951). The antigen contained 2.8 mg/ml of protein. Seventy mg of protein antigen was placed in each presterilized bottle and lyophilized before shipment to the Center for Disease Control (CDC) for immunization of rabbits.

Immunization of Rabbits

At the CDC the adult SPB antigen in each bottle was reconstituted with 5 ml of sterile distilled water to produce a stock antigen, which was further diluted with water to prepare an antigen containing 1 mg of SPB protein per kg of animal weight. Freund's incomplete adjuvant was added in equal volumes to the aqueous SPB antigen and emulsified as described by White and others, (1975). Three New Zealand white rabbits, ranging in weight from 3.8 to 5.0 kg, were immunized by footpad-subcutaneous injection (Chappell and others 1976) with enough adult SPB antigen to constitute 1 mg/kg of gross body weight (GBW). The dose of adjuvant-antigen was divided between the 2 front footpads. Ten ml of preimmunization blood was collected from the central artery of an ear of each rabbit. On the 32nd day after the initial injection, all rabbits were given booster footpad injections of 1 mg/kg GBW SPB antigen without adjuvant.

On days 11, 18, 25, 32, 39 and 46 after the initial injection of antigen, samples of blood were collected for antisera titer determinations. Sera were stored at -20°C.

We determined antiserum titers by reacting twofold dilutions (1/10, 1/20, 1/40,...1/5120) of SPB antigen stock with undiluted anti-adult SPB serum by agar gel double diffusion, using the glass slide method described by Chamberlain and Sudia (1967). The adult SPB antigen was diluted with 0.85 percent NaCl. The greatest dilution of adult SPB antigen producing a distinct precipitin line with undiluted anti-adult SPB serum was considered the titer of the antiserum.

Laboratory Testing

Individual adult *Ips grandicollis* (Eichhoff, adult *I. avulsus* (Eichhoff), adult platypodid, and SPB larva, pupa, teneral adult *I. calligraphus,* (Germar), *Dendroctonus terebrans* (Olivier), adult, and a day-old adult were crushed in 0.25 ml of 0.85 percent NaCl. The resulting suspensions were tested by agar gel double diffusion with undiluted anti-adult SPB serum.

Thanasimus dubius (F.) adults were collected in a bucket trap (Moser and Browne in press) baited with Frontalure® and hung in an active SPB infestation. *T. dubius* were stored separately in gelatin capsules until they arrived at the laboratory, where each was held in a 6.0 cm plastic Petri dish and presented with 1 or more adult SPB. Those seizing and feeding on the SPB adults were manipulated into gelatin capsules where feeding was completed. Those not feeding were held either in plastic Petri dishes or in gelatin capsules for 18-24 hours and were assumed to be starved.

Ten starved and 10 laboratory-fed *T. dubius* adults were crushed individually on a small piece of filter paper and given code numbers. Each smear was suspended overnight in 0.5 ml of 0.85 percent NaCl at 4°C. Coded aliquots of the undiluted predator suspensions were tested by agar gel double diffusion with undiluted rabbit anti-adult SPB serum.

Nineteen adult *T. dubius* were fed various numbers of SPB adults; 8 fed on 1 SPB each, 8 fed on 2 SPB each, and 3 were left overnight to feed at will. Specimens of *T. dubius* were killed and crushed on filter paper after feeding was complete. All were held over P_2O_5 at room temperature (Dempster 1960) until extracted in 0.25 ml of 0.85 percent NaCl at 4°C. An additional 20 *T. dubius* were starved to death at room temperature, then individually crushed and extracted in 0.25 ml of 0.85 percent NaCl at 4°C. These uncoded, undiluted, predator suspensions were tested in separate groups by agar gel double diffusion with undiluted anti-adult SPB serum.

Because 15 ul of predator extract might not contain enough antigen to produce a positive test, we used a concentrated predator extract to determine duration of SPB protein in the predator.

T. dubius adults, collected from baited bucket traps, were held with an excess of SPB adults for 24-48 hours in gallon plastic containers to assure removal of any other prey material from their

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digestive systems. Each *T. dubius* adult was placed in a 6.0 cm plastic petri dish and presented with 1 or more adult SPB. Those seizing and feeding on the adult SPB were manipulated into gelatin capsules. These *T. dubius* were held without feeding for 0 to 36 hours and were killed by freezing at 4-hour intervals. They were extracted as previously described and a 15 ul portion of each 0.25 ml extract was tested against undiluted homologous anti-adult SPB serum by agar gel double diffusion. The remaining 235 ul were concentrated by lyophilization, reconstituted in 15 ul of distilled, demineralized H_2O and tested as previously described.

RESULTS AND DISCUSSION

Titers for the rabbit-produced, anti-adult SPB sera reached 1/80 on day 18, and all 3 rabbit antisera reached titers of 1/160 on days 25 and 32. Downe and West (1954) indicate that acceptable results in detecting prey in a predator can be obtained with titers as low as 1/100. Although anti-SPB sera from all 3 rabbits were used in the experiments described above, the results with antiserum from rabbit No. 3 were the most consistent and are presented in table 1.

The anti-adult SPB serum is not genus specific. We obtained positive reactions to extracts of adult *I. calligraphus,* adult *I. grandicollis,* SPB larva, pupa, teneral adult, day-old adult, and adult *D. terebrans.* Downe and West (1964) and Boreham[2] found that antisera made from adult antigen of insects with paurometabolous development are sensitive to antigen from at least the older nymphal instars. Downe and West (1964) hypothesize that adult hemolymph may contain nymphal antigens and discrete adult antigens. Our results indicate that the same may be true of insects that exhibit holometabolous development.

Table 1 shows the increase in sensitivity of the anti-adult SPB serum to its homologous antigen as time passes. With day-46 anti-adult SPB serum, all 10 of the *T. dubius* adults that fed on adult SPB produced positive precipitin reactions. Seven of the 10 predators starved from 18-24 hours after feeding produced negative precipitin tests. There were 3 positive tests, #2,7, and 14. These may be the result of the more sensitive antisera reacting to small amounts of the SPB protein in the 15 ul samples from the

Table 1 — *Precipitin reactions to individual predator extracts.*

T. dubius number	Fed on SPB	Days antiserum collected after initial immunization					
		11	18	25	32	39	46
1	No	–	–	–	–	–	–
2	No	–	–	–	+	+	+
3	Yes	–	+	+	+	+	+
4	Yes	+	+	+	+	+	+
5	Yes	–	+	+	+	+	+
6	No	–	–	–	–	–	–
7	No	–	–	–	–	–	+
8	Yes	–	+	–	+	+	+
9	Yes	+	+	+	+	+	+
10	No	–	–	–	–	–	–
11	Yes	–	+	+	+	+	+
12	No	–	–	–	–	–	–
13	Yes	–	+	–	+	+	+
14	No	–	–	–	+	+	+
15	No	–	–	–	–	–	–
16	Yes	+	+	+	+	+	+
17	No	–	–	–	–	–	–
18	Yes	+	+	+	+	+	+
19	Yes	–	–	–	–	+	+
20	No	–	–	–	–	–	–

T. dubius adults that retained incompletely digested SPB material in their gut from eating adult SPB captured in the bucket trap with them. Greenstone finds that some spiders stop digesting when starved and retain detectable prey for a week.[3]

Positive reactions in tests 2 and 14 first occur with day-32 antiserum, and only with day-46 antiserum for test 7. An explanation for the delayed positive test is that, since each visible precipitin line is the result of more than one antigen-antibody system (Crowle 1973), not enough of the particular antibody required for a visible precipitin line was produced until at least days 32 or 46.

In another experiment, 19 *T. dubius* adults that had fed on either 1 or 2 SPB adults, or had fed overnight, gave positive precipitin tests in every case. When extracts of the 20 *T. dubius* adults that were starved to death were tested, no precipitin tests were positive. Control agar gel double diffusion tests with undiluted SPB antigen against homologous anti-adult SPB serum were positive in every case.

Table 2 shows the effect of time on starvation and the use of dilute and concentrated predator extractions in the agar gel double diffusion test. The number of positive tests decreases rapidly when 15 ul of predator extract are used until

[1] Use of trade names is solely for the purpose of identification and does not constitute endorsement by the U.S. Department of Agriculture or U.S. Department of Health, Education and Welfare.

[2] Dr. P. F. L. Boreham, Imperial College Field Station, Silwood Park, Ascot, Berks. SL57PY, personal communication.

[3] Dr. Matthew Greenstone, University of California, Irvine 92717, personal communication.

Table 2. – *Effect of starvation and use of partial and whole predator extracts on the agar gel double diffusion test.*

Hours Post-Feeding	No. of *T. dubius*	% Positive Tests: 15 ul extract	% Positive Tests: Concentrated Extract (235 ul)
0	12	96.6	100
4	14	85.7	100
8	12	83.3	100
12	7	57.1	100
16	7	0	100
20	15	0	100
28	10	–	100
36	8	–	75

there are no positive tests for 16 and 20 hours. Using the concentrated predator extracts, we achieved 100 percent positive tests through 28 hours after feeding. The tests were arbitrarily terminated at 36 hours when less than 100 percent results were achieved. Probably *T. dubius* adults in the field would not be without food this long (Thatcher and Pickard 1966). Negative findings for 7 of the 10 starved *T. dubius* adults in the previous experiment (table 1) are more likely the result of using a portion of the predator extract (15 ul) rather than the whole insect concentrate.

Use of a whole insect concentrate and a broad spectrum antiserum produced positive results in the controlled laboratory feeding study (table 2). Research is in progress to increase test sensitivity and produce a species specific anti-adult SPB serum.

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